

WHAT IS CLAIMED IS:

1. An engineered *Streptococcus pyogenes* Cas9 (SpCas9) protein variant comprising a mutation at two or more of amino acid positions 526, 562, 652, 661, 691, 780, 810, 848, 855, 1003, and 1060 (with reference to the numbering system of *Streptococcus pyogenes* Cas9, SpCas9), wherein a Lysine (K) at one or more of the foregoing amino acid positions is changed to a Leucine (L) or Glutamine (Q), and/or an Arginine (R) at one or more of the foregoing amino acid positions is changed to a Leucine (L) or Glutamine (Q).

2. The SpCas9 protein variant of claim 1, wherein the SpCas9 protein variant comprising a K855L/Q mutation and at least one other mutation at amino acid positions 526, 562, 652, 661, 691, 780, 810, 848, 1003, and 1060 (with reference to the numbering system of *Streptococcus pyogenes* Cas9, SpCas9).

3. The engineered SpCas9 protein variant of claim 1, wherein the SpCas9 protein variant comprising a R661L/Q mutation and at least one other mutation at amino acid positions 526, 562, 652, 691, 780, 810, 848, 855, 1003, and 1060 (with reference to the numbering system of *Streptococcus pyogenes* Cas9, SpCas9).

4. The engineered SpCas9 protein variant of claim 1, comprising two mutations at two different amino acid positions selected from K526L/Q, K562L/Q, K652L/Q, K810L/Q, K848L/Q, K855L/Q, R661L/Q, R691L/Q, R780L/Q, K1003L/Q, and R1060L/Q (with reference to the numbering system of *Streptococcus pyogenes* Cas9, SpCas9).

5. The engineered SpCas9 protein variant of claim 1, comprising three mutations at three different amino acid positions selected from K526L/Q, K562L/Q, K652L/Q, K810L/Q, K848L/Q, K855L/Q, R661L/Q, R691L/Q, R780L/Q, K1003L/Q, and R1060L/Q.

6. The engineered SpCas9 protein variant of claim 5, wherein the mutations are selected from the following group:

K562L-R661L-K855Q;
K562Q-R661L-K855Q;
K652L-R661L-K855Q;
K652Q-R661L-K855Q;
R661L-K855Q-K1003Q; and
R661L-K855Q-R1060Q.

7. The engineered SpCas9 protein variant of claim 5, wherein the mutations are selected from the following group:

K562L-R661L-K855Q;
K562Q-R661L-K855Q;
K652L-R661L-K855Q; and
K652Q-R661L-K855Q.

8. The engineered SpCas9 protein variant of claim 5, wherein the mutations are selected from the following group:

K526L-R661L-K855Q;
R661L-R691L-K855Q;
R661L-R780L-K855Q;
R661L-R780Q-K855Q;
R661L-K810L-K855Q, and
R661L-K848L-K855Q.

9. The engineered SpCas9 protein variant of claim 5, wherein the mutations are selected from the following group:

K526Q-R661L-K855Q;
R661L-K810Q-K855Q;
R661L-K855Q-K1003L; and
R661L-K855Q-R1060L.

10. The engineered SpCas9 protein variant of any preceding claim, further comprising one or more heterologous domains fused to the N terminal end, the C terminal end, an internal location, or combination thereof.

11. The engineered SpCas9 protein variant of claim 10, wherein the heterologous domain is selected from a nuclear localization signal, a cell-penetrating domain, a marker or reporter domain which facilitates detection, a chromatin modification domain, an epigenetic modification domain, a transcriptional regulation domain, a DNA or RNA deaminase domain, a uracil-DNA-glycosylase domain, a reverse transcriptase domain, a recombinase domain, an RNA aptamer binding domain, and a non-Cas9 nuclease domain.

12. The engineered SpCas9 protein variant of any preceding claim, further comprising at least one nuclear localization signal fused to the N terminal end, the C terminal end, an internal location, or combination thereof.

13. The engineered SpCas9 protein variant of any preceding claim, further comprising at least one mutation in the RuvC domain, and/or at least one mutation in the HNH domain.

14. The engineered SpCas9 protein variant of claim 13, wherein the at least one mutation in the RuvC domain, if present, comprises at least one mutation selected from D10A, D8A, E762A, and D986A; and wherein the at least one mutation in the HNH domain, if present, comprises at least one mutation selected from H840A, H559A, N854A, N856A, and N863A.

15. An engineered Cas9 system comprising the engineered SpCas9 protein variant of any preceding claim and at least one engineered guide RNA(s), wherein the at least one engineered guide RNA is designed to complex with the engineered SpCas9 protein variant.

16. A plurality of nucleic acids encoding the engineered SpCas9 protein variant of any preceding claim.

17. A plurality of nucleic acids encoding the engineered SpCas9 system of claim 15.

18. The plurality of nucleic acids of claim 17, the plurality of nucleic acids comprising at least one nucleic acid encoding the engineered SpCas9 protein variant and at least one nucleic acid encoding the engineered guide RNA.

19. The plurality of nucleic acids of any one of claims 16-18, wherein at least one nucleic acid is RNA.

20. The plurality of nucleic acids of any one of claims 16-18, wherein at least one nucleic acid is DNA.

21. The plurality of nucleic acids of any one of claims 16-20, wherein the at least one nucleic acid encoding the engineered SpCas9 protein variant is codon optimized for expression in a eukaryotic cell.

22. The plurality of nucleic acids of claim 21, wherein the eukaryotic cell is a human cell, a non-human mammalian cell, a non-mammalian vertebrate cell, an invertebrate cell, a plant cell, or a single cell eukaryotic organism.

23. The plurality of nucleic acids of claim 15, wherein the at least one nucleic acid encoding the engineered guide RNA is DNA.

24. The plurality of nucleic acids of claim 15, wherein the at least one nucleic acid encoding the engineered SpCas9 protein variant is operably linked to a phage promoter sequence for *in vitro* RNA synthesis or protein expression in a bacterial cell, and the at least one nucleic acid encoding the engineered guide RNA is operably linked to a phage promoter sequence for *in vitro* RNA synthesis.

25. The plurality of nucleic acids of claim 12, wherein the at least one nucleic acid encoding the engineered Cas9 protein variant is operably linked to a eukaryotic promoter sequence for expression in a eukaryotic cell, and the at least one nucleic acid encoding the engineered guide RNA is operably linked to a eukaryotic promoter sequence for expression in a eukaryotic cell.

26. At least one vector comprising the plurality of nucleic acids of any one of claims 16-25.

27. The at least one vector of claim 26, which is a plasmid vector, a viral vector, or a self-replicating viral RNA replicon.

28. A eukaryotic cell comprising at least one engineered Cas9 system of claim 15 or the nucleic acid of claims 17 or 18.

29. The eukaryotic cell of claim 28, which is a human cell, a non-human mammalian cell, a plant cell, a non-mammalian vertebrate cell, an invertebrate cell, or a single cell eukaryotic organism.

30. The eukaryotic cell of claim 29, which is *in vivo*, *ex vivo*, or *in vitro*.

31. The engineered SpCas9 protein variant of any of claims 1-13, which is a Cas9 homologue.

32. A ribonucleoprotein (RNP) complex comprising the engineered SpCas9 protein variant of any of claims 1-13.

33. A fusion protein comprising the engineered SpCas9 protein variant of any of claims 1-13.

34. A pharmaceutical composition comprising the engineered SpCas9 protein variant of any of claims 1-13 and at least one pharmaceutically acceptable excipient.

35. An in vitro method for modifying the chromosomal sequence of a eukaryotic cell, the method comprising expressing in the eukaryotic cell the engineered SpCas9 protein variant of any of claims 1-13 together with a guide RNA.